The characterisation of synthetic and natural-product pharmaceuticals by electrospray ionisation-mass spectrometry (ESI-MS) and liquid chromatography (LC)-ESI-MS

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This article considers the application of topical analytical techniques – electrospray ionisation-mass spectrometry (ESI-MS) and liquid chromatography (LC)-ESI-MS – to the characterisation of selected small molecular-mass synthetic and natural-product pharmaceuticals, as well as bioactive peptides. We chose the synthetic drugs according to selected structural classes in which they give ESI signals primarily as $[M + H]^+$ ions. The structural classes chosen to illustrate the application of ESI-MS and LC-ESI-MS to such drug characterisation are drugs with amine-containing side chains, drugs with *N*-containing saturated ring structures, and 1,4-benzodiazepines and other heterocyclic hypnotics. We then discuss potential natural-product pharmaceuticals, such as selected quinolines and nicotines. We next apply the technique to the characterisation of molecules of unknown structure present in plant extracts or fractions that possess antibacterial activity. Finally, we discuss the isolation and the characterisation of bioactive peptides from frog-skin secretions utilising an arsenal of MS tools as well as Edman degradation sequencing and cDNA cloning.
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1. Introduction

Electrospray ionisation-mass spectrometry (ESI-MS) was introduced by Yamashita and Fenn [1] in 1984 with drugs and their related molecules being subjected to increasing investigation by this soft-ionisation technique. The Royal Swedish Academy of Sciences awarded The Nobel Prize in Chemistry for 2002 partly to John B. Fenn for his pioneering work in ESI-MS.

Detailed structural information on drugs can be obtained by resort to techniques such as cone-voltage fragmentation with a single MS instrument, collision-induced dissociation (CID) with triple-quadrupole MS instruments and MS^n techniques using quadrupole ion-trap instrumentation.

ESI is now the most important ionisation technique for the on-line coupling of liquid-phase separation techniques, such as liquid chromatography (LC) with MS. The Web of Knowledge database reveals 29, 35, 55, 72 and 87 publications in the years 2001, 2002, 2003, 2004 and 2005, respectively, on the LC-ESI-MS of drug molecules. Perusal of these database publications shows how LC-ESI-MS is being used for the analysis of a wide variety of drug molecules in matrices such as body

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fluids, foods, natural waters, and pharmaceutical formulations.

This article considers the application of the analytical techniques ESI-MS and LC-ESI-MS to the characterisation of selected small molecular mass synthetic and natural-product pharmaceuticals, as well as bioactive peptides. The synthetic drugs are chosen according to selected structural classes in which they give ESI signals primarily as $[M + H]^+$ ions.

The structural classes chosen to illustrate the application of ESI-MS and LC-ESI-MS to such drug characterisation are drugs with amine-containing side chains, drugs with *N*-containing saturated ring structures and 1,4-benzodiazepines/other heterocyclic hypnotics.

We then discuss potential natural-product pharmaceuticals, such as selected quinolines and nicotines.

The article continues with the application of the technique to the characterisation of molecules of unknown structure present in plant extracts/fractions that possess antibacterial activity.

Finally, we discuss the isolation and the characterisation of bioactive peptides from frog-skin secretions utilising an arsenal of MS tools as well as Edman degradation sequencing and cDNA cloning.

2. Drugs with amine-containing side chains

Joyce et al. [2] investigated the characterisation of selected drugs with amine-containing side chains using ESI-MS^n. From this study, certain rules were formulated with respect to the ESI-MS^n behaviour of drugs with amine-containing side chains. For example, the synthetic opiate agonist methadone has the following structure:

$H_3C–CH_2–CO–C(C_6H_5)_2–CH_2–CH(CH_3)–N(CH_3)_2$

It therefore has a carbon chain ending in a tertiary nitrogen atom with at least two methylene or substituted methylene groups separating this nitrogen atom from the other end of the carbon chain. Such a molecule, according to [2], will first lose the end nitrogen atom as the corresponding secondary amine, $HN(CH_3)_2$, followed by loss of the corresponding alkene, $CH_2{=}CHCH_3$, formed from these two methylene/substituted-methylene groups. Such structural information from ESI-MS^n, obtained sequentially for this and other drugs with amine-containing side chains and supported by electrospray ionisation-quadrupole time of flight-mass spectrometric (ESI-QToF-MS^2) elemental analyses, contrasts with that from the hard ionisation technique, electron impact-mass spectrometry (EI-MS), which, for methadone, gives no molecular ion at *m/z* 309, a single base peak at *m/z* 72 and several small signals of less than 5% relative abundance. If there is only one methylene group adjoining the end nitrogen atom, as is the case with lignocaine, then the end of this carbon chain becomes the detectable charged species, as in $CH_2{=}N^+(C_2H_5)_2$, and no neutral amine and alkene is formed [2].

These MS^n experiments, supported by ESI-QToF-MS^2 data, therefore show certain characteristic fragmentations with respect to the amine-containing side chains. The data therefore provide useful information on the structure of these compounds with amine-containing side chains and can be used in the characterisation of such drugs and their structurally-related metabolites. The ESI-MS^n data of such compounds can be held in a database and neutral mass losses/low molecular mass ions cross-referenced with such data obtained from analytes of unknown structure, which can then be of value in their structural characterisation with respect to those molecules with amine-containing side chains. A table giving such mass losses/signals at low *m/z* values in the range *m/z* 15–176 for drugs studied in our laboratory was presented in a review [3], which will be of value in the characterisation of unknown metabolites and natural-product pharmaceuticals isolated from plants, for example. Table 1 is a sample table for the range *m/z* 17–42 taken from [3] and featuring some of the molecules discussed in this article.

The ESI-MS^n and LC-ESI-MS^2 of selected antidepressant drugs, some with amine-containing side chains (i.e. citalopram, fluoxetine, sertraline and venlafaxine) has recently been investigated in our University of Ulster Coleraine (UUC) laboratory [4]. Following elucidation of the fragmentation mechanisms using ion-trap (IT)-MS, supported by ESI-QToF-MS^2 measurements, these molecules can be unambiguously identified and determined in mixtures at low-ng/ml concentrations by the application of LC-ESI-MS^2, as illustrated in Fig. 1.

Saliva is increasingly being used for drug testing to monitor illicit and licit drug use. Saliva is a natural ultrafiltrate of plasma with molecules transported across epithelial membranes. Highly protein-bound drugs are unlikely to cross the cellular membranes, so saliva testing offers the possibility of direct comparison of unbound, pharmacologically-active drug concentrations with the observed effects. Improved technology, such as LC-ESI-MS, makes it possible to make many diagnoses from saliva analysis that are currently made from blood analysis. Collection of saliva is safe and patient-friendly and requires no qualified personnel, and that could make it an important diagnostic matrix of the future. Four of the drugs with amine-containing side chains studied [2] (i.e. amphetamine, clenbuterol, flurazepam and methadone) have been simultaneously identified and determined in saliva samples at low-ng/ml concentrations by the application of LC-ESI-MS [2].

Table 1. Mass losses/signals at low *m/z* values using ESI-MS and structural inferences (Reprinted from [3], © 2005, with permission from Elsevier)

Mass loss/signal at low *m/z value*	Structural inference	Examples
17	Loss of NH_3 from end-of-chain NH_2 group with at least 2 adjacent CH_2 groups	Amphetamine, 5-HT
17	Loss of NH_3 from pyrrolidine ring	Nornicotine
17	Loss of NH_3 from aromatic NH_2 group	2,6-Xylidine(lignocaine metabolite)
18	Loss of H_2O from aliphatic OH	Clenbuterol, salbutamol, morphine, codeine, 3-OH-benzodiazepines, quinolines, yohimbine
20	Loss of HF	7-Aminoflunitrazepam, *N*-desmethylflunitrazepam
28	Loss of $CH_2{=}CH_2$ after loss of adjacent end-of-chain amine	Chlorpromazine
28	Loss of $CH_2{=}CH_2$ from ethyl ester substituent	Pethidine
28	Loss of CO/NCH_2 with ring contraction	Benzodiazepines, zopiclone, coumarins, quinolines
29	Loss of $COH/CH_2{=}NH$ with ring contraction	Benzodiazepines e.g., flunitrazepam
31	Loss of CH_3NH_2 from end-of-chain	N'methyl 5-HT
31	Loss of CH_3NH_2 from CH_3-substituted pyrrolidine ring	Nicotine
31	Loss of OCH_3	Coumarins
32	Loss of S from cyclic structure	Chlorpromazine
32	Loss of CH_3OH from methyl ester substituent	Cocaine, reserpine
35/36	Loss of aromatic Cl/HCl	Clenbuterol, benzodiazepines
42	Loss of $CH_3CH{=}CH_2$ after loss of end-of-chain amine	Methadone trimpramine
42	Loss of C_3H_6 side-chain	Coumarins
42	Loss of $CH_3CH{=}CH_2$ from -NH-$CH(CH_3)_2$ end-of-chain group	Propranol
42	Loss of ketene, $CH_2{=}C{=}O$	Coumarins, 7-acetamidonitrazepam

Hair samples are also being increasingly studied for drug detection and determination at typically low-ng/mg hair concentrations in pharmaceutical compliance testing and forensic toxicology. On 31 January 2000, Dr. Harold Shipman was convicted at Preston, England, of murdering 15 of his patients by administering lethal doses of diamorphine (pharmaceutical heroin). Gas chromatography (GC)-MS and LC-MS hair analyses were presented as evidence and formed the cornerstone of the prosecution case.

The ESI-MS^n characterisation of selected anti-psychotic drugs with amine-containing side chains, such as chlorpromazine and trifluperazine, and their detection and determination in human hair samples by LC-ESI-MS^2 was investigated by McClean et al. [5]. Possible mechanisms for the observed MS^n-fragmentation patterns were proposed. A validated LC-ESI-MS^2 method was then applied to the detection and determination of these drugs in the hair of a patient under clinical treatment for schizophrenia. Chlorpromazine, trifluoperazine and flupenthixol were identified and determined in this hair sample, following alkaline degradation of the matrix, solvent extraction and LC-ESI-MS^2 using trimipramine as internal standard. For example, the limit of detection (LOD) for chlorpromazine using this technique was found to be 18 μg/L and this drug was found at a concentration of 1.24 ng/mg in the hair sample. From the viewpoint of a rival analytical methodology, GC-MS analysis of veterinary tranquillisers in urine has given a slightly lower LOD of 5 μg/L for chlorpromazine [6].

3. Drugs with nitrogen-containing saturated ring structures

The ESI-MS^n of selected drugs with nitrogen-containing saturated ring structures, such as piperidines and piperazines, was investigated by Smyth et al. [7]. Sequential product-ion fragmentation experiments (MS^n) were performed in order to elucidate the degradation pathways for the $[M + H]^+$ ions and their predominant fragment ions. These MS^n experiments show certain characteristic fragmentations in that functional groups are generally cleaved from the ring systems as neutral molecules, such as H_2O, amines, alkenes, esters, and carboxylic acids. When such a nitrogen-containing drug molecule also contains a functional group, such as an ester, that, on liberation as a neutral molecule, has a significantly higher negative ΔH_f^0 value than that of the corresponding amine, then the ester is preferentially liberated. This is illustrated in Fig. 2 for pethidine with Table 2 showing the heat of formation of some of the neutral molecules formed in fragmentations following ESI of drug molecules. Furthermore, when an aromatic entity is present in these drug molecules together with the nitrogen-containing saturated ring structure, fragmentation

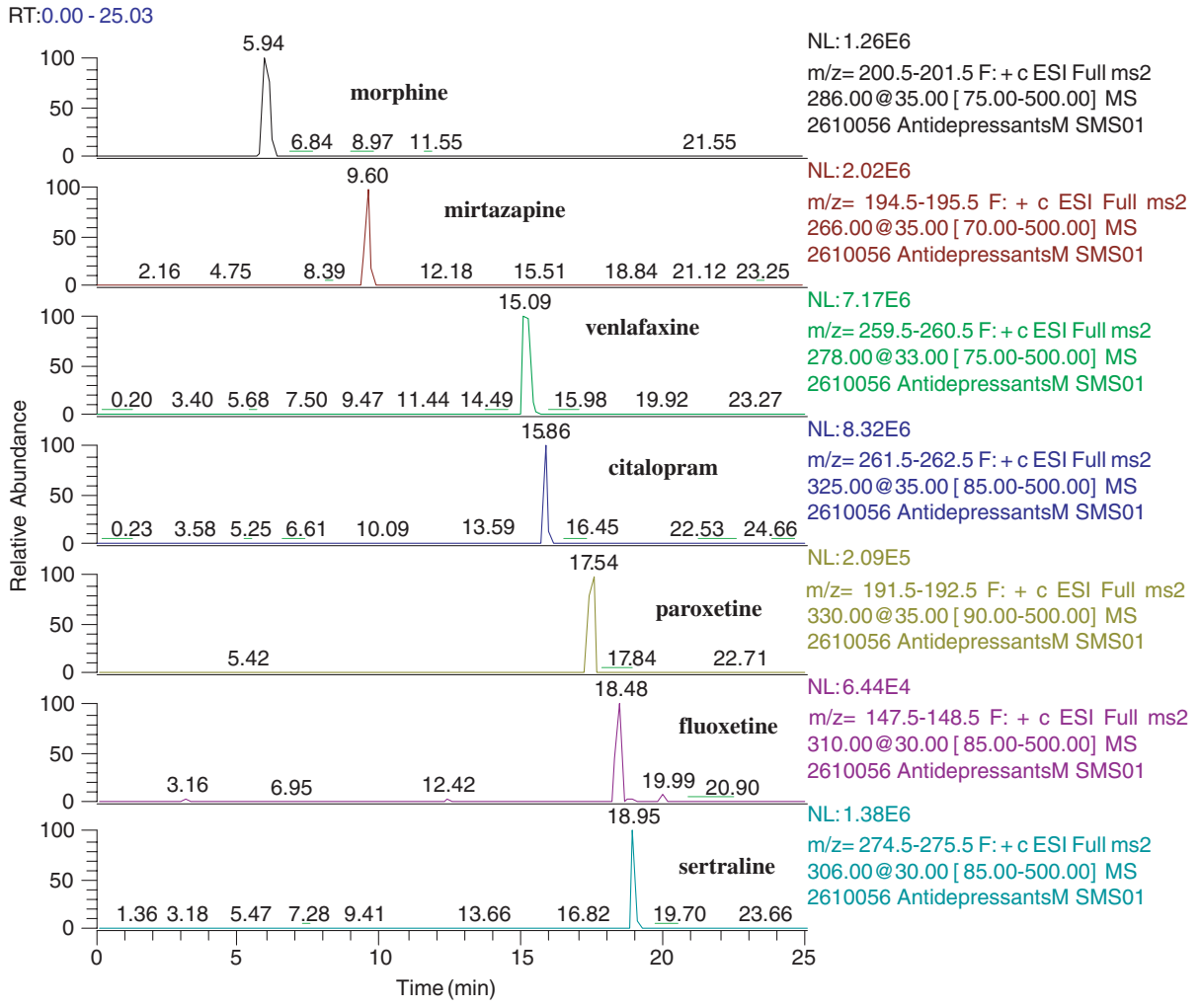

Figure 1. LC-ESI-MS^2 chromatogram of six antidepressant drugs using morphine as an internal standard.

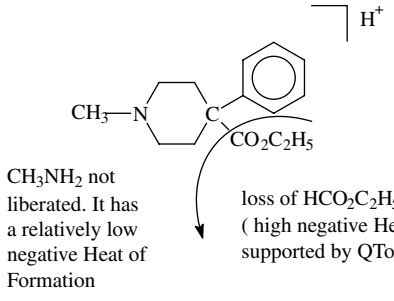

Figure 2. Fragmentation process for pethidine using ESI-MS^2. (Reprinted from [3], © 2005, with permission from Elsevier).

occurs to the latter ring with the former being predictably resistant to fragmentation. The structures of fragment ions proposed for ESI-MS^n can be supported by ESI-QToF-MS^2. The data presented [7] therefore provided useful information on the structure of these heterocyclic compounds, which could be used to characterise synthetic drug metabolites and unidentified natural-product pharmaceuticals isolated from natural sources.

Table 2. Heat of formation of neutral molecules formed in fragmentations following ESI of drug molecules (Reprinted from [3], © 2005, with permission from Elsevier)

Neutral molecule	ΔH_f^0 (kJ/mol)
HF(g)	−271.1
HCl(g)	−92.3
H_2O(g)	−241.8
SO_2(g)	−296.8
NH_3(g)	−46.1
NO_2(g)	+33.2
CO(g)	−110.5
CO_2(g)	−393.5
C_5H_{12}(g)	−146.4
$CH_3COOC_2H_5$(l)	−486.6
CH_3NH_2(g)	−28.0
$C_2H_5NH_2$(g)	−48.5
C_6H_5OH(s)	−165.0

4. Flunitrazepam (Rohypnol) and other heterocyclic hypnotics

LC-ESI-MS can be used for identification and quantitation of flunitrazepam with concentrations in the range 0.5–20 ng/mL for parent drug and metabolites being determinable (e.g., in a date-rape case, the marker metabolite, 7-aminoflunitrazepam was determined at 11.9 ng/mL in urine and 0.9 ng/mL in plasma using automated SPE and LC-ESI-MS with SIM of the protonated molecular ions [8]).

It should be noted that after LC separation as neutral molecules on a C_{18} column with mobile phase 65% MeOH, 5% AN & 30% H_2O, these drug molecules arrive at the MS detector as the protonated species. It can be surmised that they become protonated in the ESI process by protons produced in the electrospray capillary, i.e.

$$2H_2O(l) = O_2(g) + 4H^+(aq) + 4e^-$$

Alternatively, the drug molecules can become protonated by protons present as impurities in the mobile-phase solvents (e.g., AN contains 0.002% CH_3-COOH).

The ESI-MS^n of selected hypnotic drugs (i.e. zopiclone, zolpidem, flunitrazepam and their metabolites (Fig. 3)) has been investigated [9]. Again, these MS^n experiments show certain characteristic fragmentations, in that functional groups are generally cleaved from the ring systems as neutral molecules, such as H_2O, CO, CO_2, amines and HF. These molecules have relatively high negative heat-of-formation values, which facilitates their liberation as neutral molecules in the fragmentation

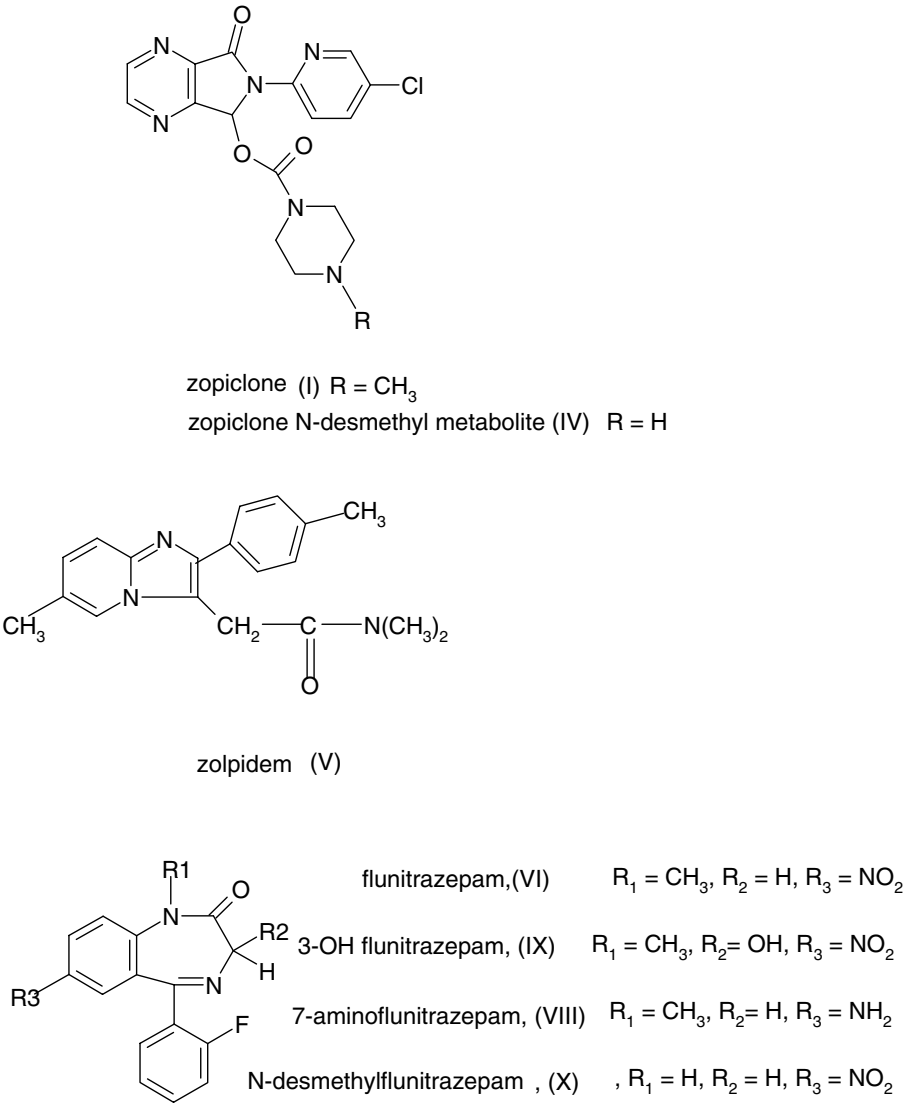

Figure 3. Molecular structures of zopiclone, zolpidem, flunitrazepam and their metabolites (Reprinted from [3], © 2005, with permission from Elsevier).

processes (Table 2). Again, when an aromatic entity is present in a drug molecule together with a nitrogen-containing saturated ring structure, as with zopiclone and its *N*-desmethyl metabolite, fragmentation initially occurs at the latter ring with the former being predictably resistant to fragmentation. The structures of fragment ions proposed for ESI-MS^n have been supported by ESI-QToF-MS^2. These hypnotics have been identified and determined in mixtures at low-ng/ml concentrations by the application of LC-ESI-MS^n, which has been used for their analysis in saliva samples [9].

5. Quinolines

Fragmentation mechanisms of quinoline alkaloids of significance in plants have been investigated using ESI–MS^n with a view to characterisation of molecules of unknown structure isolated from these natural sources [10]. This investigation has led to the generation of an appropriate database incorporating data from ESI-MS^n and also from GC and LC for these low molecular mass quinolines and has been put to practical application for the identification of quinoline alkaloids in a plant extract. Thus, an acid extraction of the leaves of *Choisya ternata* containing such tertiary alkaloids was analysed by LC-ESI-MS and the resulting behaviour of the quinolines was compared with that of the quinoline alkaloids in the database.

Mass spectral data for all the LC peaks were recorded, as can be seen in Table 3, and some of the proposed structures are given in Fig. 4. For the peaks numbered 1, 2, 5 and 7, the $[M + H]^+$ ion is observed at *m/z* 330, which indicates a molecule with a molecular mass of 329. This corresponds to choisyine, a compound known to be present in this plant. From database information obtained under the same conditions the retention time of choisyine is 6.9 min. Peak number 5 elutes at 6.95 min, indicating that this peak could be due to choisyine. Peaks 1, 2 and 7 may be due to the presence of isomeric

Table 3. HPLC-ESI-MS results for the acid soluble extract of *Choisya ternata*

Peak number	Retention time	$(M + H)^+$
1	3.48	330
2	4.93	330
3	5.79	348
4	6.43	348
5	6.95	330
6	7.80	260
7	8.96	330
8	9.48	366
9	12.95	344
10	15.06	313
11	16.64	296
12	20.54	328

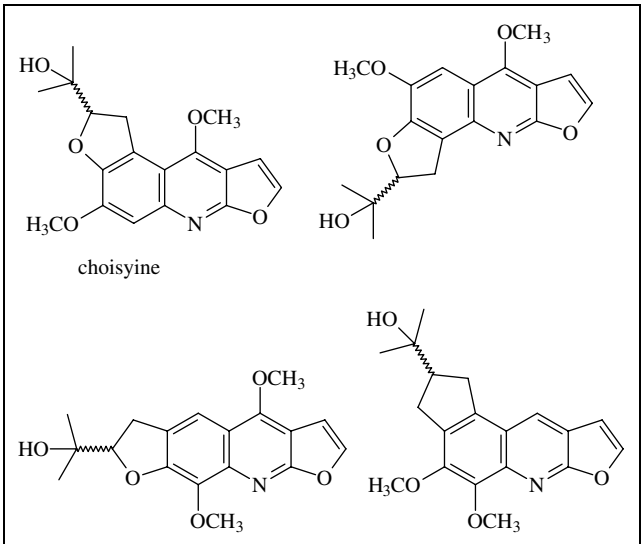

Figure 4. Some Quinoline derivatives isolated from *Choisya ternate*.

structures of choisyine, such as those given in Fig. 4. This would account for the same $[M + H]^+$ ion but different retention times for these molecules. One of these isomeric structures has already been isolated from *Choisya ternata*. Similarly, the analytes that elute giving peak numbers 3 and 4 have a molecular mass of 347. Comparison with database information indicates that peak number 3 may be due to evoxine as the retention times correspond. Peak number 4 could then be due to an isomeric structure of evoxine. Similar deductions can be made about the identities of the remaining LC peaks.

The development of the database of quinoline alkaloids and synthetic derivatives therefore helps in the identification of quinoline alkaloids present in plant extracts by comparing the GC and LC retention times, *m/z* values of $[M + H]^+$ ions and fragmentation pathways by MS^n. It could also help to establish biogenic pathways of quinoline alkaloids by identifying precursors that are normally present in very small quantities in the plant extracts.

6. Nicotines

An ESI-MS^n study of the fragmentation of the alkaloid nicotine and its metabolites/degradation products is being carried out by Smyth et al. [11]. The fragmentation patterns of nicotine (1) and nine related molecules are being investigated using both ESI-MS^n and Q-ToF-MS^2: nicotine-1-N-oxide (2), nicotine-1′-N-oxide (3), nicotine-1,1′-di-N-oxide (4), β-nicotyrine (5), nornicotine (6), anatabine (7), myosmine (8), cotinine (9), and anabasine (10). Such characterisation is important in the quality control of nicotine-containing active pharmaceutical ingredients (APIs) produced by the local firm,

Nicobrand Limited, for use in anti-smoking therapy products.

As an example of ESI-MS^n behaviour, nicotine $C_{10}H_{14}N_2$ gives its pseudomolecular ion, $[M + H]^+$, at m/z 163.2 and it fragments at MS^2 to give a major signal at m/z 132.1 and a lesser one at m/z 106.1. The loss of 31u is attributed to the loss of CH_3NH_2 from the saturated heterocyclic ring rather than from the relatively stable pyridine entity. The fragment ion at m/z 106.1 is due to the loss of the neutral molecule, C_2H_2. The product ion at m/z 132.1 at MS^3 loses H_2 to give a signal at m/z 130.2. This is illustrated in Fig. 5 and this fragmentation is supported by QToF-MS^2 elemental analysis.

When oxidised, nicotine can be converted to cotinine ($C_{10}H_{12}N_2O$), the main metabolite of nicotine and a marker molecule for tobacco-smoke exposure. It gives an $[M + H]^+$ ion at m/z 177.2. On fragmentation at MS^2, three product ions were produced, – at m/z 146.2 (C_9H_8NO), m/z 98 (C_5H_8NO) and m/z 80 (C_5H_6O) – with this fragmentation being supported by QToF-MS^2 elemental analysis, as shown in parenthesis. The major product ion was at m/z 146.2, which was the loss of CH_3NH_2 from cotinine. This ion further fragmented to give a signal at m/z 118.1, due to the removal of a molecule of carbon monoxide.

Previous methods for the separation of nicotine and its metabolites and degradation products used non-volatile buffers that were unsuitable for LC-ESI-MS analysis. As a result, an analytical method has been developed [11] for use with LC-ESI-MS and LC-UV for the characterisation and the quantification of nicotine and its metabolites and degradation products down to levels of 0.01%. Fig. 6 shows this characterisation by both LC-ESI-MS and LC-UV for these nicotines.

7. ESI-MS^n characterisation of biologically active extracts of Australian bush medicines

The preceding sub-sections have dealt with the characterisation by ESI-MS and HPLC-ESI-MS of synthetic and natural-product pharmaceuticals of known molecular structure. This sub-section considers how far such techniques can help in the characterisation of natural-product pharmaceuticals of unknown molecular structure in extracts and fractions taken from plants of Australian origin. The nature of the research has involved extraction of plant parts with organic solvent, chromatographic fractionation, bioassay of the fractions against strains of disease causing bacteria

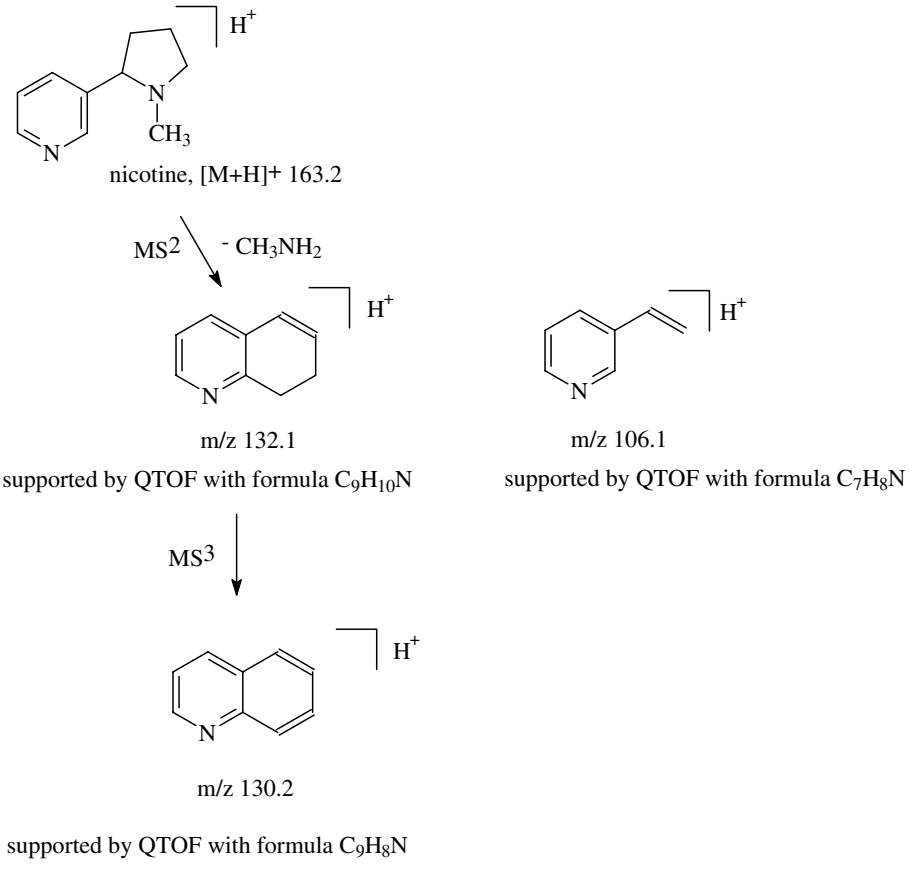

Figure 5. MS^n fragmentation pattern for nicotine.

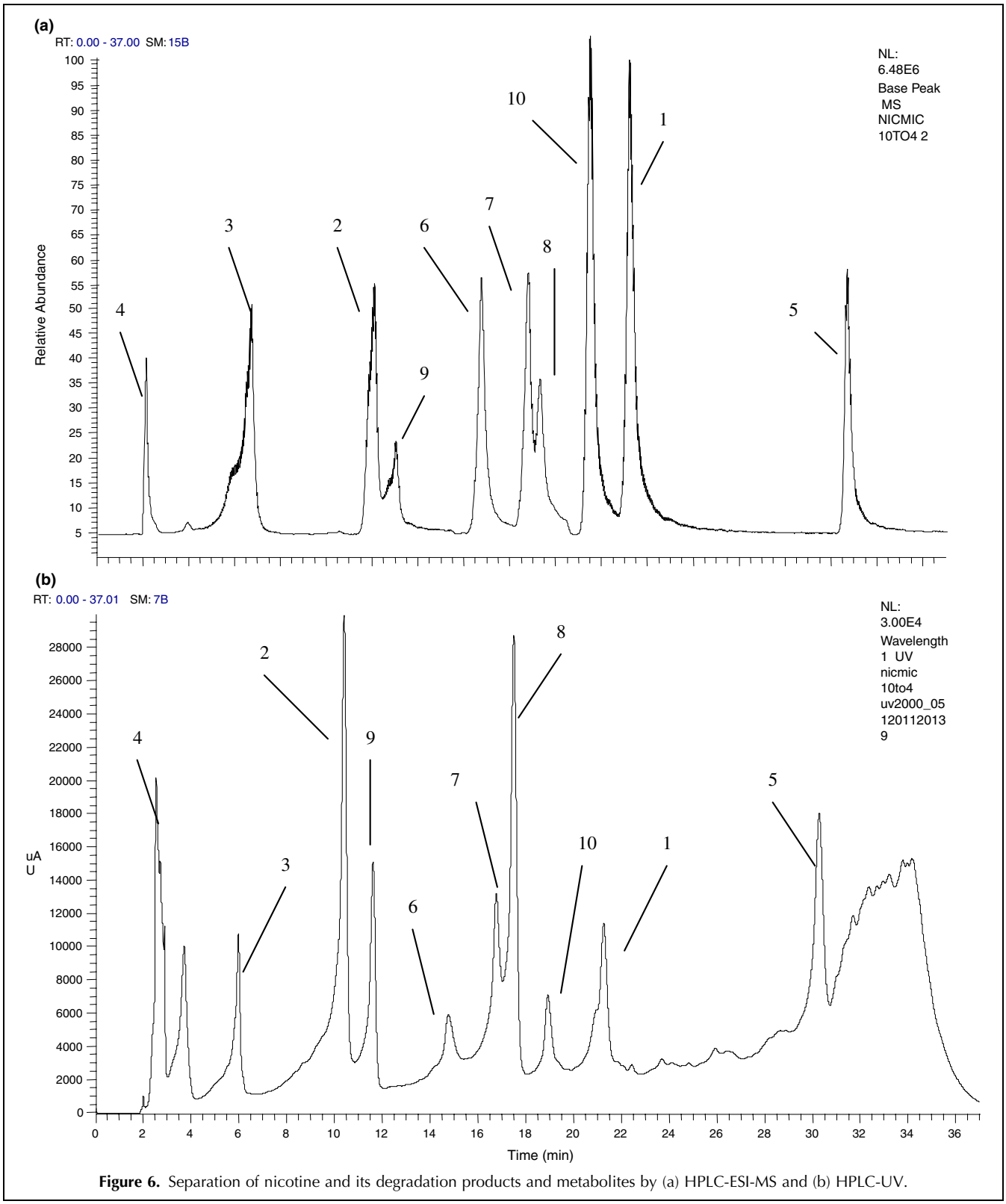

Figure 6. Separation of nicotine and its degradation products and metabolites by (a) HPLC-ESI-MS and (b) HPLC-UV.

and investigation of active fractions by ESI-MS. Ultimately, the goal is identification of novel antimicrobial compounds that may become new pharmaceutical compounds.

We next discuss an example of ESI-MS characterisation of a preparative thin-layer chromatography (TLC) fraction from a methanolic extract of the leaves of a particular Australian plant. This biologically-active

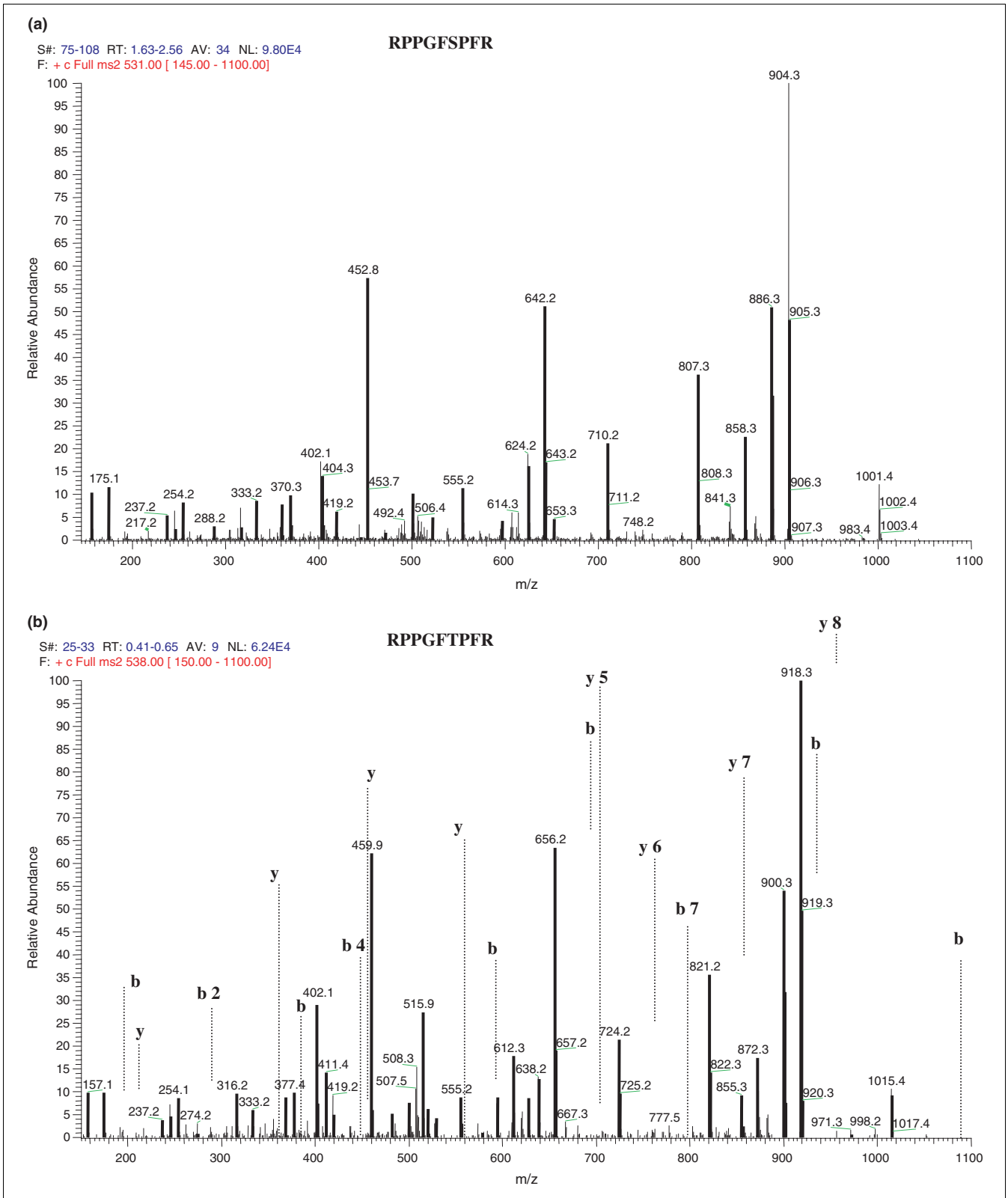

Figure 7. MS^2 spectra of doubly-charged ions of bradykinin ($m/z = 531$) and threonine-6 bradykinin ($m/z = 538$). Identified b-ion and y-ion fragment series used in sequence calls are indicated in each case. (Reprinted from [20], © 2002, with permission from Elsevier).

fraction gave a major $[M + H]^+$ ion at m/z 403, which gave product ions at m/z 335 and 371 on application of MS^2. This corresponds to 68u and 32u losses respectively. The latter loss could be due to CH_3OH from a methyl-ester substituent, as has been observed for cocaine and reserpine (Table 1), and the 68u loss due to a

C_5H_8 side chain, as has been observed for coumarins [12,13] and also quinolines.

The ion at *m/z* 335 further fragmented at MS^3 to generate a major ion at *m/z* 219, a loss of 116u that could be due to $C_6H_{16}N_2$ or $C_6H_{12}O_2$ on application of the MS nitrogen rule. This latter loss could involve a substantial change to the structure of the original molecule so that further MS^n studies would be of little benefit. It is therefore not possible to fully structurally identify this molecule in this biologically active fraction by ESI-MS^n. ESI-QToF-MS^2 will yield an elemental analysis of the unknown molecule, preparative LC will isolate of the order of mg of the active molecule and high-resolution nuclear magnetic resonance (NMR) spectrometry will fully ascertain its structure.

8. ESI-MS characterisation of biologically-active peptides from frog venoms

The defensive skin secretions of exotic amphibians contain diverse biologically-active compounds, including biogenic amines, peptides, proteins, steroidal bufadienolides and cardenolides [14]. In the UUC laboratory, the ESI-MS^n behaviour of indole alkaloids found in the Australian Golden Bell frog *Litoria aurea* has been studied [15]. However, the main focus of the research programme was the isolation and the characterisation of bioactive peptides from frog-skin secretions utilising an arsenal of MS tools as well as Edman degradation sequencing and cDNA cloning.

For a number of years, attention has been paid to the characterisation of peptides in frog venom. *Xenopus laevis* is an example of one frog found to contain antimicrobial peptides that may limit the growth of bacteria on the skin of amphibians and also help to combat wound infection. Antibacterial peptides have also been isolated from the European red frog, *Rana temporaria*. Some of these peptides have been found to exhibit haemolytic activity [16]. Characterisation of other peptides has shown their similarity to neurotransmitters or hormones of mammalian origin, such as caerulein/cholecystokinin, bradykinin, tachykinins and thyrotropin-releasing hormones [17].

Secretions have been obtained from in-house captive frogs using mild electrical stimulation of the moistened dorsal skin surface. The resulting milky venom was then rinsed from the frog's back with distilled water and lyophilised to produce a white fluffy powder. Such harvesting resulted in milligrams of dried material that were then subjected to chromatographic sub-fractionation followed by bioactivity assays and structural elucidation of the active constituents.

In the UUC laboratory, bradykinin-related peptides have been characterised from the defensive skin secretions of Bombina frogs. Following chromatographic separation of *Bombina orientalis* venom, peptides exhibiting signals at *m/z* 531 and 538, representing the $[M + 2H]^{2+}$ ions of bradykinin and threonine-6 bradykinin, respectively, were detected by ESI-MS^n. These precursors were subjected to fragmentation in the QqQ-IT and the resulting MS^2 profile submitted to the Sequest algorithm (Thermo, San Jose, Ca, USA) for processing using the non-redundant FASTA database. The amino-acid sequences of these peptides were then verified as bradykinin (RPPGFSPFR) and its threonine-6 variant (RPPGFTPFR). Figs. 7a and 7b show these patterns and the resulting b-ion and y-ion assignment for the fragments produced.

Such MS methods of structural elucidation provide a fast, efficient approach for screening large numbers of peptides in a relatively short period of time, particularly when the protein sequences are in readily available databases. For novel peptides, MS^2 fragmentation patterns have been generated using QToF-MS that facilitated the use of *de novo* peptide sequencing to establish the b-ion and y-ion series for a particular peptide without recourse to a database of sequences.

For biological activity assays, synthetic analogues of bradykinin and threonine-6 bradykinin were produced using FMOC solid-phase peptide synthesis. These produced dose-dependent relaxation of rat arterial smooth muscle and constriction of intestinal smooth muscle. Further details of these pharmacological experiments are available [18].

This strategy of ''peptide prospecting'' has been found to be successful in isolating other novel bradykinin peptides [19] and classes of peptides that have insulin-releasing activity [20–23].

Acknowledgements

The authors thank Nicobrand for the provision of nicotine and nine related molecules that were investigated using both ESI-MS^n and Q-ToF-ESI-MS^2: nicotine-1-N-oxide, nicotine-1′-N-oxide, nicotine-1,1′-di-N-oxide, β-nicotyrine, nornicotine, anatabine, myosmine, cotinine and anabasine.

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